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**Concurrent Oral 9 - rheumatoid arthritis: aetiopathogenesis [OP59-OP64]:
OP59. The value of interleukin-17 serum level in rheumatoid arthritis
immunopathogenesis**

Biliavska, I V ; Bortkevych, O P

Abstract: Background: Interleukin (IL)-17 is the main Th-1 cytokine, produced by activated T-lymphocytes. The potential IL-17 value in rheumatoid arthritis (RA) pathogenesis consists of its independent inflammatory response induction and mediated stimulation of proinflammatory factors synthesis resulting in joint destruction. The aim of study was to determine the role of IL-17 in immuno-inflammatory/autoimmune reactions development and to reveal IL-17 serum level associations with clinical and immunological characteristics of RA. Methods: 50 patients with early RA (disease duration < 12 month) and 15 healthy individuals were examined. All patients underwent complex clinical and laboratory examination. The immunological investigation included lymphocyte count and its subpopulative composition determination using monoclonal antibodies; detection of circulative immune complex level was done by Digeon method, IgG, IgM, IgA - by Mancini method. The serum concentrations of CRP(DAI, USA), sCD40L, matrix metalloproteinases (MMP)-3 (Bender MedSystems, Austria), IL-17 (Biosource, Belgium), rheumatoid factor (RF) («Vektorbest», Russia), anti-CCP antibodies (Axies-Shield Diagnostic, UK) were revealed using ELISA immunoassay. Results: On the base of IL-17 serum level patients were divided in two groups: group1 (n = 28) were patients with normal IL-17 serum level and group2 (n = 22) were those with high IL-17 serum level. In the group2, the rate of patients' pain assessment by visual analogue scale (67.3 ± 7.2 vs 32.8 ± 4.6 ; $P < 0.001$), tender (16.7 ± 2.0 vs 8.4 ± 1.1 ; $P < 0.01$) and swollen (12.3 ± 2.3 vs 3.9 ± 0.8 ; $P < 0.01$) joint count, DAS28 (5.0 ± 0.4 vs 2.8 ± 0.2 $P < 0.01$) were significantly higher compare to group1. It was found that in group2 the higher T-lymphocyte amount (CD3) was due to CD4 higher quantity, at the same time CD8 amount was significantly lower ($22.2 \pm 1.5\%$ vs $28.4 \pm 1.7\%$, $P < 0.05$) compare to group1. This caused the immunoregulative index increasing and indicated in the lost of autoimmune process regulation, including B-lymphocytes (CD19) activation. The CD154 expression was significantly lower in the group2 ($3.4 \pm 0.4\%$ vs $10.8 \pm 2.8\%$, $P < 0.05$) compare to group1. The difference in autoimmune reaction indices wasn't significant between groups except antibody-producing B-lymphocytes ($13.7 \pm 1.5\%$ vs $8.5 \pm 1.0\%$, $P < 0.05$) and IgM RF serum level (2.9 ± 0.3 U/ml vs 1.6 ± 0.5 U/ml, $P < 0.05$), which were significantly higher in group1. The IL-17 level had a positive correlative connections with DAS28 ($r = 0.7$; $P < 0.05$), circulative immune complex level ($r = 0.38$; $P < 0.05$), anti-CCP antibodies ($r = 0.4$; $P < 0.05$), IgM RF ($r = 0.41$; $P < 0.05$), CD4 ($r = 0.38$; $P < 0.05$) and negative correlative connection with CD8 ($r = -0.39$; $P < 0.05$). Conclusions: The importance of IL-17 value in immuno-inflammatory and autoimmune reactions development through T-lymphocytes activation in RA pathogenesis was confirmed. Thus the influence on T-depended immuno-inflammatory reaction products synthesis could be a new therapeutic target of RA patients' management. Disclosure statement: All authors have declared no conflicts of interest

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BSR CONCURRENT ORAL PRESENTATION OF ABSTRACTS

Concurrent Oral 9 – Rheumatoid Arthritis: Aetiopathogenesis

OP59. THE VALUE OF INTERLEUKIN-17 SERUM LEVEL IN RHEUMATOID ARTHRITIS IMMUNOPATHOGENESIS

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Background: Interleukin (IL)-17 is the main Th-1 cytokine, produced by activated T-lymphocytes. The potential IL-17 value in rheumatoid arthritis (RA) pathogenesis consists of its independent inflammatory response induction and mediated stimulation of proinflammatory factors synthesis resulting in joint destruction. The aim of study was to determine the role of IL-17 in immuno-inflammatory/autoimmune reactions development and to reveal IL-17 serum level associations with clinical and immunological characteristics of RA.

Methods: 50 patients with early RA (disease duration < 12 month) and 15 healthy individuals were examined. All patients underwent complex clinical and laboratory examination. The immunological investigation included lymphocyte count and its subpopulative composition determination using monoclonal antibodies; detection of circulative immune complex level was done by Digeon method, IgG, IgM, IgA - by Mancini method. The serum concentrations of CRP(DAI, USA), sCD40L, matrix metalloproteinases (MMP)-3 (Bender MedSystems, Austria), IL-17 (Biosource, Belgium), rheumatoid factor (RF) («Vektorbest», Russia), anti-CCP antibodies (Axies-Shield Diagnostic, UK) were revealed using ELISA immunoassay.

Results: On the base of IL-17 serum level patients were divided in two groups: group1 (n=28) were patients with normal IL-17 serum level and group2 (n=22) were those with high IL-17 serum level. In the group2, the rate of patients' pain assessment by visual analogue scale (67.3 ± 7.2 vs 32.8 ± 4.6 ; $P < 0.001$), tender (16.7 ± 2.0 vs 8.4 ± 1.1 ; $P < 0.01$) and swollen (12.3 ± 2.3 vs 3.9 ± 0.8 ; $P < 0.01$) joint count, DAS28 (5.0 ± 0.4 vs 2.8 ± 0.2 $P < 0.01$) were significantly higher compare to group1.

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Conclusions: The importance of IL-17 value in immuno-inflammatory and autoimmune reactions development through T-lymphocytes activation in RA pathogenesis was confirmed. Thus the influence on T-dependent immuno-inflammatory reaction products synthesis could be a new therapeutic target of RA patients' management.

Disclosure statement: All authors have declared no conflicts of interest.

OP60. DEFECTIVE CD3+CD8+CD28 SUPPRESSOR FUNCTION IN RA PATIENTS IS PARTIALLY RESTORED AFTER ANTI-TNF α INHIBITOR THERAPY AND MAY BE ASSOCIATED WITH REDUCED ICOS AND PD-1 EXPRESSION

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Background: Immunoregulatory mechanisms in rheumatoid arthritis (RA) are present but may be deficient and unable to suppress persistent inflammation in the joints. CD3+CD8+CD28-T cells have been shown to have suppressor function which may be compromised in autoimmune disease. Previously we have demonstrated that the function of CD3+CD8+CD28- cells is deficient in RA patients. In this study, we aimed to determine whether treatment by tumour necrosis factor alpha (TNF α) inhibitors may re-establish their defective regulatory function.

Methods: Heparinized blood samples from age-matched healthy controls (HC), osteoarthritis (OA) patients, RA patients on methotrexate (RAMTX) and RA patients on anti-TNF α therapy (RATNF) were collected and four colour flow cytometry was performed to determine the percentage of CD3+CD8+CD28- cells in the peripheral blood. Functional characterization of the CD3+CD8+CD28- cells followed negative isolation of these cells using immunomagnetic beads (purity = 97%). The cells were cocultured with autologous peripheral blood mononuclear cells (PBMC) at the ratio 1:1 in the presence of anti-CD3 antibody for 72 h and cell proliferation was determined by tritiated thymidine incorporation. The surface expression of ICOS and PD-1 on CD3+CD8+CD28- cells was determined after 48 h stimulation with anti-CD3 antibody.

Results: RAMTX patients showed significantly increased percentages of CD3+CD8+CD28 cells ($68 \pm 7\%$, n=46) compared with OA patients ($55.4 \pm 15\%$, n=17, $P=0.05$), HC ($45.3 \pm 8\%$, n=15, $P=0.04$) and RATNF patients ($47.4 \pm 15\%$, n=21, $P=0.04$). Functional assays demonstrated that the addition of CD3+CD8+CD28 cells to autologous PBMC (ratio 1:1) resulted in significant suppression of PBMC proliferation to anti-CD3 antibody stimulation in HC ($45.3 \pm 11\%$ inhibition, $P=0.03$) but not in the RAMTX cultures ($1.5 \pm 2\%$ inhibition). In contrast, RATNF CD3+CD8+CD28 cells significantly suppressed PBMC proliferation ($30 \pm 19\%$ inhibition, $P=0.05$). Further investigation of the mechanism whereby RAMTX cells fail to suppress showed that significantly fewer cells in the anti-CD3 stimulated CD3+CD8+CD28-cell population from RAMTX patients expressed ICOS ($2.0 \pm 2\%$, RAMTX vs $8.7 \pm 3.2\%$, HC; n=10 RAMTX/HC, $P < 0.0001$) and PD-1 ($2.3 \pm 3\%$, RAMTX vs $9.5 \pm 2\%$, HC; $P < 0.0001$). Similar studies for RATNF patients are ongoing with preliminary data indicating that in some RATNF patients, the expression of PD-1 is partially restored ($5.4 \pm 3\%$; n=5).

Conclusions: This study indicates that although the percentage of CD3+CD8+CD28 cells is higher in RAMTX patients, they have deficient suppressor function, which may be restored following TNF α inhibitor therapy. This impaired suppressor activity may be related to cell surface expression of ICOS and PD-1.

Disclosure statement: All authors have declared no conflicts of interest.

OP61. RHEUMATOID SYNOVIAL FIBROBLASTS SUPPORT AID EXPRESSION AND IG CLASS-SWITCHING IN B CELLS VIA A BAFF-DEPENDENT TLR3-STIMULATED PATHWAY

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Background: Rheumatoid arthritis (RA) is characterized by the presence of synovial niches of autoreactive B cells which express activation-induced cytidine deaminase (AID), the enzyme initiating Ig class-switching (CSR) and sustain in situ autoantibody. Importantly, B cell niches remain functional in the RA-SCID model in the absence of recirculating cells, suggesting that autocrine mechanisms support ongoing B cell activation in the RA synovium. Here we investigated whether RA synovial fibroblasts (RASf), which are known to contribute to RA synovitis, are capable of directly regulating B cell activation, AID

expression and CSR. In addition, we dissected the molecular basis of stromal cell/B cell interactions with particular emphasis on the role of toll-like receptors (TLRs) signaling and B cell survival/proliferation factors.

Methods: mRNA and protein expression of B cell survival factors BAFF and APRIL in RASF and OASF stimulated with TLR2, TLR3 and TLR4 ligands was assessed by Taqman PCR (QT-PCR) and ELISA, respectively. Un-switched IgD⁺ B cells were isolated from human tonsils using magnetic cell sorting. Isolated B cells were co-cultured via transwell or cell-cell contact with RASF/OASF for 24 h and 72 h in the presence or absence of TLR ligands and with or without BCMA-Ig as a blocker of soluble BAFF/APRIL. AID mRNA expression and IgM/A/G production were measured to assess functional activation of B cells. In addition, γ -C μ and λ -C μ circular transcripts (CT, molecular by-products of ongoing CSR from IgM to IgG and IgM to IgA, respectively) were assessed by rt-PCR.

Results: In vitro stimulation of TLR3 and to a significantly lesser extent TLR4, but not TLR2 on RASF led to strong induction of BAFF (~1,000-fold increase with TLR3) and APRIL mRNA expression. In response to TLR3, BAFF was time-dependently released in the supernatant of RASF (~300 pg/ml) and, to a lesser extent, OASF. TLR3 stimulation of RASF in co-culture with B cells strongly enhanced AID expression, ongoing CSR to IgG, but not IgA, as shown by detection of γ -C μ CT and release of IgG. By contrast, TLR3 stimulation alone had no direct effect on B cells. Conversely, blockade of soluble BAFF/APRIL by BCMA-Ig inhibited TLR3-induced RASF-dependent production of AID mRNA, γ -C μ CT as well as the secretion of IgG.

Conclusions: We demonstrated that RASF are able to release high levels of B cell survival factors upon TLR3 stimulation at both mRNA and protein level. The release of these factors was functional, as demonstrated by the capacity of RASF to directly modulate AID expression, CSR and production of class-switched antibodies in co-cultured un-switched B cells. This effect was abrogated by blockade of soluble BAFF and APRIL. Overall, these data strongly support a fundamental role for TLR3-dependent release of BAFF and APRIL by RASF in sustaining functional B cell activation and antibody production.

Disclosure statement: All authors have declared no conflicts of interest.

OP62. QUANTIFYING IN VIVO FLUORESCENCE IMAGING IN MURINE ARTHRITIS BY TARGETING E-SELECTIN

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Background: In vivo molecular optical imaging can delineate at the macroscopic level biologic processes that are occurring at the cellular and molecular level. E-Selectin, a leucocyte adhesion molecule expressed on activated endothelium, is upregulated by TNF α and increased in RA. Furthermore, radiolabelled F(ab')₂ fragment of anti-E-Selectin monoclonal antibody demonstrates increased specificity compared with conventional technetium-oxidronate scanning in patients with RA. Collagen-induced arthritis (CIA), an animal model of RA, has been widely used to study the pathogenesis of arthritis and to identify new therapies for RA, including anti-TNF α . Pro-inflammatory cytokines, such as TNF α and IL-1 β , are expressed in the arthritic joints of both murine CIA and RA. This study aimed to demonstrate and quantify E-Selectin targeted fluorescent imaging *in vivo* in a model of paw inflammation following local injection of murine TNF α and subsequently in CIA as a model of RA.

Methods: Anti-murine E-selectin and isotype control antibodies were cultured from hybridoma cell lines and labelled with Dylight 750 nm near-infra red probe (excitation/emission spectra of 752 nm/778 nm). Specificity of antibody binding to recombinant murine E-selectin pre- and post-labelling was confirmed by ELISA. Paw inflammation was induced by the intraplantar injection of murine TNF (50 ng). CIA was induced by an intradermal injection of bovine type II collagen (100 μ g) emulsified in complete Freund's adjuvant. Following injection of labelled antibodies animals were imaged using a Kodak FX Pro Optical *in vivo* imaging system.

Results: Mean fluorescence intensity was measured over the time course of TNF α -induced inflammation and demonstrated a 1.98 fold increase ($P < 0.01$) in signal at 8 h following injection of anti-E-selectin antibody compared with isotype control in inflamed paws. E-selectin signal was significantly abrogated by pre-treatment with etanercept ($P < 0.05$ vs untreated). Fluorescence was also measured in the in

CIA model and showed a reduction in E-Selectin mean fluorescent signal intensities from 11220 ± 2225 ($n = 4$) in mice with established arthritis, to 6188 ± 787 ($n = 6$; $P < 0.05$) following etanercept treatment. Immunohistochemical analyses confirmed that E-Selectin expression can be detected following induction of arthritis in the CIA model at early, mid and late time points of arthritis.

Conclusions: Targeted *in vivo* optical imaging in TNF α induced paw oedema is a quantifiable molecular imaging technique that delineates increased signal due to E-selectin upregulation in the inflamed paw. The TNF inhibitor etanercept significantly abrogated signal in CIA. The differential distribution of E-Selectin targeted signal into inflamed tissue compared with non-specific IgG may enhance drug targeting. Novel hardware technology and highly innovative reporter probes and dyes have potential for translating into future molecular imaging techniques for patients with arthritis.

Disclosure statement: All authors have declared no conflicts of interest.

OP63. TSG-6: AN AUTOCRINE REGULATOR OF INFLAMMATORY JOINT DISEASE?

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Background: TSG-6, the secreted product of TNF-stimulated gene 6, is present in the cartilage, synovium and synovial fluids of RA patients. It is constitutively expressed by RA synoviocytes, where its production is up-regulated by TNF α , IL-1 and IL-17. Animal models indicate a protective role for TSG-6 in inflammatory joint disease, with evidence of both anti-inflammatory and chondroprotective effects in mice with collagen- and antigen-induced arthritis. Recent *in vitro* studies have indicated an additional anti-resorptive effect of TSG-6, possibly via an interaction with RANKL, the major regulator of osteoclastogenesis. TSG-6 has a similar potency to OPG (the soluble decoy receptor for RANKL) but, unlike OPG, inhibits only osteoclast activation and not formation. The fully-humanized monoclonal antibody against RANKL, denosumab, has been shown to reduce erosion scores in clinical trials of RA, but its utility may be diminished by its lack of anti-inflammatory properties. This study sought to determine whether (i) TSG-6 acts synergistically with OPG in inhibiting osteoclastic bone resorption, (ii) TSG-6 is produced during cytokine-mediated osteoclastogenesis and (iii) TSG-6 can regulate the process of osteoclastogenesis in patients with inflammatory joint disease.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers; synovial fluid (SF) macrophages were derived from patients with inflammatory and non-inflammatory joint disease and synovial tissue samples were derived from biopsy and arthroplasty specimens. PBMCs and SF macrophages were cultured in the presence or absence of inflammatory mediators (TNF α , IL-1, IL-6 and IL-17) \pm TSG-6 and \pm OPG. Osteoclast formation was assessed by TRAP staining and osteoclast activity by lacunar resorption. TSG-6 release in cell culture media by osteoclast precursors and mature osteoclasts was measured using ELISA. TSG-6 expression in synovial tissue was assessed immunohistochemically.

Results: Our data show that:

1) TSG-6 is expressed by osteoclasts and in the synovium of RA patients; 2) TSG-6 and OPG have synergistic effects on the inhibition of RANKL-mediated bone resorption; 3) the inflammatory cytokines TNF α , IL-1 and IL-6 but not IL-17 induce TSG-6 production by osteoclast precursors (PBMCs) and 4) induction of TSG-6 expression in PBMCs correlates with an inhibition of osteoclast-mediated lacunar resorption, suggesting an autocrine control of osteoclast activity by TSG-6.

Conclusions: In the presence of inflammatory cytokines, osteoclast precursors produce TSG-6 at concentrations that are sufficient to inhibit osteoclast activity and reduce lacunar resorption. This may represent an autocrine mechanism to limit the degree of bone resorption during joint inflammation. Although in the complex milieu of inflammatory synovial fluid, this mechanism is insufficient to prevent local erosion, the chondroprotective, anti-inflammatory and anti-resorptive effects of TSG-6 make it a potential therapeutic option.

Disclosure statement: All authors have declared no conflicts of interest.

OP64. OSTEOCLAST-MEDIATED BONE RESORPTION: REGULATION BY HYPOXIA-INDUCIBLE FACTOR (HIF) AND ANGIOPOIETIN-LIKE 4 (ANGPTL4)

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Background: Hypoxia is a feature of the hyperplastic synovium in rheumatoid arthritis (RA). Many cellular components of RA express the hypoxia-inducible transcription factor, HIF. We have recently demonstrated that osteoclast-mediated bone resorption is enhanced by hypoxia in a HIF-1 α -dependent manner (1). We continue our investigation of the molecular mechanisms regulating hypoxia-induced osteoclast activation to further understanding of bone resorption in RA.

Methods: Osteoclasts were differentiated from CD14+ PBMC with M-CSF (25 ng/ml) and RANKL (50 ng/ml) for 16 days. Osteoclasts were then exposed to hypoxia (2% O₂) for 24 h prior to fixation, analysis of resorption (toluidine blue staining of dentine slices) or collection of RNA, protein or cell supernatant. To identify potential genes of interest, an Illumina HumanWG-6 v3.0 48k array was performed comparing 6 paired samples of normoxic vs hypoxic osteoclasts. Transfection of mature osteoclasts with siRNA against HIF-1 α or HIF-2 α was performed using RNAiMAX (Invitrogen).

Results: Use of a panel of normoxic inducers of HIF (CoCl₂, desferrioxamine, dimethylxalyl glycine, L-mimosine) revealed that HIF expression is sufficient to enhance osteoclast resorption in the absence of a hypoxic stimulus. Analysis of microarray data therefore focussed on known HIF target genes.

ANGPTL4 is a HIF-regulated gene and putative endocrine signalling molecule involved in lipid and glucose metabolism. A potential role in osteoclast resorption was postulated based on known associations with MMP expression and cartilage degradation (2) and over-expression in RA (3). We have confirmed time- and O₂ concentration-dependent upregulation of ANGPTL4 in osteoclasts and osteoblasts by real-time PCR, Western blotting and ELISA. ANGPTL4 expression was also increased by normoxic inducers of HIF. Exogenous ANGPTL4 (25–100 ng/ml) caused a 2.5-fold increase in osteoclastic bone resorption. It could also partially correct the inhibition of hypoxic induction of osteoclast activity caused by transfection of mature osteoclasts with HIF-1 α siRNA. However, ANGPTL4 had no effect on either osteoclast differentiation from monocytic precursors, or on monocyte survival / proliferation.

Conclusions: These data demonstrate that expression of HIF is sufficient to stimulate osteoclast-mediated bone resorption in the absence of a hypoxic stimulus. ANGPTL4 is at least partially able to compensate for HIF-1 α deficiency with respect to stimulation of osteoclast activity. This is the first description of a role(s) for ANGPTL4 in osteoclast biology and represents a potential mechanism whereby HIF could regulate bone resorption in the RA microenvironment.

Disclosure statement: All authors have declared no conflicts of interest.

autoimmune sialoadenitis, to characterize the cellular and molecular basis of autoreactive B cell activation and evolution of functional Ectopic Lymphoid Structures (ELS) in the chronically inflamed NOD salivary glands.

Methods: Submandibular glands from 110 female NOD mice from 4 to 35 weeks of age were collected. Paired snap-frozen samples were analysed by immunohistochemistry (IHC) for T and B lymphocytes (CD3/CD20) to evaluate cell infiltration and the degree of B/T cell segregation. ELS were detected by staining for FDC-M1 (follicular dendritic cell networks), GL7 (germinal centre B cells) and AID (marker for ELS functionality). Characterization of B cell subsets within the infiltrates was carried out by immunostaining and by FACS analysis with CD19, CD21, CD23, B220, IgD, IgM, CD1d and CXCR5 antibodies. Quantitative TaqMan real-time PCR was performed to investigate the mRNA expression of ELS-related genes. Sex/age matched Balb/c and C57BL/6 mice were used as controls.

Results: NOD infiltrates in glands displayed progressive features of ELS from week 8, with 75% of mice developing B/T cell segregation, FDC networks and GL7+ ectopic germinal centers from week 20. Evolution of TLSs was closely associated with mRNA upregulation of genes regulating ELS organization and function such as lymphoid chemokines CXCL13/CCL19 and their receptors CXCR5/CXCR7, lymphotaxins and B cell survival factors BAFF and APRIL. In agreement with CXCL13/CXCR5 mRNA expression, B cells in infiltrates display strong CXCR5 expression and were mostly characterized by a follicular phenotype (B220+/IgD+/IgMlow/CD23+/CD21low) as demonstrated by both IHC and FACS analysis on isolated cells. Finally, functionality of ELS was demonstrated by expression of AID mRNA and protein within FDC networks, which paralleled the detection of circulating SS-related autoantibodies.

Conclusions: This work provided the first in-depth characterization of cellular and molecular mechanisms underlying the evolution of functional TLSs within submandibular infiltrates of NOD mice. These data strongly support the hypothesis that B-cells can be activated within TLSs in the target organ and promote *in situ* autoantibody response. Overall, these data support the critical importance of ELS formation in chronic autoimmune inflammation and identified NOD mice as a suitable model to test therapeutic strategies aimed at modulating B cell functionality.

Disclosure statement: All authors have declared no conflicts of interest.

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OP66. INDIRECT COSTS ESTIMATION IN PRIMARY SJÖGREN'S SYNDROME

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Background: The aim of this study was to estimate the indirect costs, such as loss of time from work, associated with primary Sjögren's syndrome (pSS) compared with Rheumatoid Arthritis (RA) and community controls.

Methods: Data were obtained from 84 female patients with pSS as part of a study to develop a systemic activity measure, from 87 consecutive female patients with RA attending a hospital clinic and from 96 female community controls on a general practice list. A modified economic component of the Stanford Health Assessment Questionnaire was used to assess lost productivity. Indirect costs

Concurrent Oral 10 – Connective Tissue Disease

OP65. MOLECULAR AND CELLULAR EVOLUTION OF FUNCTIONAL TERTIARY LYMPHOID STRUCTURES IN SALIVARY GLANDS OF NOD MICE

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Background: Tertiary Lymphoid Structures (TLSs) are common features of chronic inflammatory diseases including Sjögren's syndrome (SS). We recently showed that these ectopic structures acquire secondary lymphoid organs properties and are capable of supporting B cell activation and autoantibody production including expression of activation-induced cytidine deaminase (AID) and Ig class switching. Dissecting TLSs dynamics in humans is technically and ethically challenging. Thus, we used the NOD mouse, a spontaneous model of